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(54) Title: ADENOVIRUS-MEDIATED PRODUCTION OF BIOACTIVE PROTEINS BY MAMMALIAN CELLS AND ANIMALS

(57) Abstract

The invention provides a method for producing bioactive compounds useful for therapeutic therapy in humans and non-human animals. Said compounds are produced by an adenoviral vector containing a DNA encoding an amino acid sequence for a protein. The DNA sequence is operative linked to a promoter and is injected into culture mammalian cells or into animals. Said vector produces a protein which is then post-translationally modified by the cultured mammalian cells or the animal to produce a bioactive compound. The bioactive compound is isolated from the cultured cells, or animal fluid, cells or organs.

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Adenovirus-mediated production of bioactive proteins by mammalian cells and animals.

Field of the Invention

The present invention relates generally to the field of production of bioactive molecules. More particularly, the present invention relates to a novel process to generate bioactive proteins using an adenoviral vector and cultured cells and tissues of animals.

Background of the Invention

Basic science, medicine, agriculture and various manufacturing arts use bioactive proteins to perform a variety of operations that range from treating disease to processing foodstuffs. More complex bioactive molecules such as enzymes and growth factors require complex biosyntheses that can only be performed with the cellular biosynthetic machinery. To accomplish complex biosyntheses of bioactive proteins a variety of recombinant DNA protein expression arts have been developed. An early method used expression vectors such as plasmids or bacterial viruses to transform common bacteria for production of the protein of interest. Although this method is still in use, it has three major limitations: (i) the newly generated recombinant protein may not be compatible with survival of the host bacterium or the cell may destroy the protein; (ii) the protein may be precipitated within the bacterium in an insoluble form leading to a difficult or impossible recovery of protein; and (iii) the host bacterium usually lacks the proper biosynthetic machinery for post-translational modification and the protein fails to function like the native protein. To avoid these problems eukaryotic systems have been devised. The most

prevalent system used today is the baculovirus system based upon the insect baculovirus and cultured insect cells. While many of the drawbacks inherent in the bacterial systems are avoided in the baculovirus systems, it is still limited because insect cells may not have appropriate biosynthetic machinery for post-translational modifications. Virally-based expression systems have the major draw-back that useful mutant viral strains that express recombinant proteins may cause cytopathic effects. This requires significant safety precautions in using the virus.

Summary of the Invention

An object of the present invention is a novel method of producing bioactive molecules in cultured mammalian cells using an adenoviral based vector.

Thus, in accomplishing the foregoing object there is provided in accordance with one aspect of the present invention a method of producing bioactive molecules, comprising steps of: introducing an adenoviral vector directly into cultured mammalian cells. The vector is comprised of the following elements linked sequentially at appropriate distance for functional expression: a promoter, a 5' mRNA leader sequence; an initiation site; a nucleic acid cassette containing the sequence to be expressed; a 3' untranslated region; and a polyadenylation signal. The cultured mammalian cells produce the protein coded for by the nucleic acid cassette and subsequently modify the protein so that it is bioactive. The modified protein is either released into the cell growth media

or stored within the cell. The bioactive protein is retrieved by standard protein isolation procedures.

In an alternative embodiment an adenoviral vector is introduced directly into animals. The vector is comprised of the following elements linked sequentially at appropriate distance for functional expression: a promoter, a 5' mRNA leader sequence; an initiation site; a nucleic acid cassette containing the sequence to be expressed; a 3' untranslated region; and a polyadenylation signal. The tissues of the animal produce the protein coded for by the nucleic acid cassette and subsequently modify the protein so that it is bioactive. The modified protein is either released into the fluid of the animal (serum, milk, urine, etc.) or stored within the cells and organs. The bioactive protein is retrieved by standard protein isolation processes.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention which are given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows a schematic representation of the construction of recombinant adenoviral vectors containing bioactive factor genes.

Figures 2 and 3 are Western blots showing examples of the efficient production of the bioactive molecules by human cells after transduction with recombinant

adenoviral vectors. The Western blot in Figure 2 shows that cells transduced with ADV-BDNF released BDNF into the medium that was molecularly equivalent to authentic BDNF (arrow). The Western blot in Figure 3 shows that cells transduced with ADV-CNTF made CNTF that was molecularly equivalent to authentic CNTF (arrow).

Figure 4 shows the measurement of a bioactive molecule by human cells after they were transduced with an adenoviral vector containing a nucleic acid cassette coding for a bioactive molecule. The height of the bar represents the relative amount of neuronal protective factor. The bars on the left show known amounts of the neuronal protective factor NGF. The bars on the right show the amount of NGF in 1, 5, and 15 μl of conditioned medium removed from cultures of human cells that were transduced with ADV-NGF. Very high amounts of NGF are produced and released into the surrounding medium by the transduced cells.

Figures 5 A and B show an example of the biological effect of an recombinant adenoviral vector containing the nucleic acid cassette for a bioactive molecule on rat pheochromocytoma cells (PC12 cells).

Figure 6 shows the neuronal protective effect of the product of recombinant adenoviral vectors containing nucleic acid cassettes coding for bioactive molecules on embryonic chicken sensory cells.

Figure 7 shows Western blot analyses of conditioned medium from HeLa cells transduced with Adv.RSV-*nf*. The media and cell extract contained

proteins that co-migrated (lanes 2) with the corresponding recombinant growth factors used as standards (lanes 1). Neither untransduced HeLa cells or cells transduced with Adv.RSV-βgal produced proteins that cross-reacted with the anti-NF antibodies. Cells transduced with Adv.RSV-GDNF produced multiple cross-reacting proteins (arrowheads) that indicate that the GDNF may have been post-translationally modified.

Figure 8. Biological activity of NF produced by HeLa cells transduced with Adv.RSV-*nf* vectors *in vitro*. Primary cultures of dissociated NG (panel A) and DRG (panel B) cells from chick embryos were grown in media containing commercial recombinant NF (hatched bars) or containing 25% v/v of culture medium from HeLa cells transduced with Adv.RSV-*nf* (black bars) or Adv.RSV-βgal (cross-hatched gray bar). (B+G = BDNF and GDNF; B+C+N = BDNF, CNTF, and NGF)

Detailed Description of the Invention

The term "bioactive molecule" as used herein refers to a biologically active peptide gene product that has effects on cells or systems in the mammal. Other terms used to describe bioactive molecules are: hormones, bioactive peptides, enzymes, growth factors, trophins, neurotrophins, neural growth factors, nerve growth factors, trophic factors, survival factors, and neurotrophic factors. A large number of bioactive molecules are known that include Fibroblast Growth Factor (FGF), Endothelial Growth Factor (VEGF), angiostatin, Epithelial Growth Factor (EGF), Vasopressin, Insulin, Hepatocyte Growth Factor, Insulin-Like Growth Factor (IGF), cytokines, Human

Growth Factor (HGF), Nerve Growth Factor (NGF), Brain Derived Growth Factor (BDNF), Ciliary Neurotrophic Factor (CNTF), Glial Cell Line-Derived Growth Factor (GDNF), Neurotrophin-3 (NT-3) and Neurotrophin 4/5 (NT-4/5).

The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell. A vector contains multiple genetic elements positionally and sequentially oriented, i.e., operationally linked with other necessary elements such that the nucleic acid in a nucleic acid cassette can be transcribed and when necessary, translated in the transfected cells. In the present invention, the preferred vector comprises the following elements operationally linked for functional expression; a promoter, a 5' mRNA leader sequence; an initiation site; a nucleic acid cassette containing the sequence to be expressed; a 3' untranslated region; and a polyadenylation signal.

Various promoters may be used to drive the vector useful in the method of the present invention. Representative examples of a useful promoter include Rous Sarcoma Virus - Long Terminal Repeat, Cytomegalovirus promoter, and promoters specific to the bioactive molecules or the producer cells.

The term "nucleic acid cassette" as used herein refers to the genetic material of interest which can express a protein, or a peptide, or RNA. The nucleic acid cassette is operationally linked, i.e., positionally and sequentially oriented in a vector,

with other necessary elements such that the nucleic acid in the cassette can be transcribed and, when necessary, translated. The product of the nucleic acid cassette may be further modified by the cell's protein modification processes.

The term "stable transformation" as used herein refers to transformation in which the introduced therapeutic nucleic acid sequences is not incorporated into the chromosomes of the whole cell. This leads to apparent stable change or transformation of a given characteristic of a cell.

The term "transformed" as used herein refers to a process for making or introducing a stable change in the characteristics (express phenotype) of a cell by the mechanism of gene transfer whereby DNA or RNA is introduced into a cell in a form where it expresses a specific gene product or alters an expression affects endogenous gene products. The vector can be introduced into the cell by a variety of methods including microinjection, $CaPO_4$ precipitation, lipofection (liposome fusion), use of a gene gun and DNA vector transporter. Vectors based upon the adenovirus are highly efficient in transformation of mammalian cells and far exceed the efficiency of the methods listed above.

The term "post-translational modification" as used herein refers to a process in which polypeptide chains produced by translation in the cell are modified in order to make them biologically active. These modifications require complex biological processes that

are innate to the producer cells. Examples of post-translational modification are: cleavage, glycosylation, folding, dimerization, phosphorylation, acetylation, and methylation.

The term "recombinant adenoviral vector" as used herein refers to a vector comprised of the elements described above and the genetic material derived from the adenovirus that has been rendered replication-defective by genetic engineering. The adenoviral vector is capable of infecting cells at a high efficiency but is incapable of replicating. It directs the production of the protein or proteins coded in the nucleic acid cassette.

In one embodiment of the present invention there is provided a method of producing or manufacturing bioactive molecules. This method comprises introducing an adenoviral vector directly into cultured cells.

Another embodiment of the present invention is a method of producing or manufacturing bioactive molecules in the tissues of animals. This method comprises a step of introducing an adenoviral vector directly into the animal used to produce the bioactive molecule.

Whether the bioactive molecules are produced in cultured mammalian cells or in an animal the step after the introduction of the adenovirus and production and post-translation modification of the protein is to harvest the bioactive molecule. One skilled in the art recognizes that the method of harvesting depends upon

how the protein is produced and released by the cell or animal. If the cultured mammalian cells secrete the protein into the cell growth media, then the cell growth media can be collected and standard protein isolation procedures can be used to isolate the protein for use. If the cell, itself, retains the modified protein then the cells can be harvested and again the protein is recovered by standard protein isolation procedures. In the case of animal cells the modified protein after being modified could be found in body fluids including the serum, blood, secreted milk, urine or other bodily fluids. The fluid is collected and the protein is isolated by standard protein isolation procedures. Alternatively the protein could be accumulated in the cells and tissues of the animal. In this case the animal will have to be sacrificed and the protein isolated from the tissues.

In the methods of the present invention the adenovirus vector is comprised of the following elements operationally linked for functional expression; a promoter, a 5' mRNA leader sequence; an initiation site; a nucleic acid cassette containing the sequence to be expressed; a 3' untranslated region; and a polyadenylation signal.

The adenoviral vector is preferably replication defective so that most viral proteins are not produced while the bioactive molecules are produced. The adenoviral vector is rendered replication-defective by removing essential genes required for replication and the cloned bioactive molecule genes are inserted into this region such that synthesis is regulated by a

functional promoter site. The ability to stably transduce producer cells at a high efficiency and to grow the transduced cells for days to months permits the generation of bioactive protein at high efficiency. The ability to stably transduce animals with cloned genes at a high efficiency and the production of bioactive molecules from cloned genes in adenovirus vectors in animals permits the generation of bioactive protein at high efficiency. Further the cells or animals permit the post-translational modification of the protein by the modification mechanisms native to the producer cells or the animals. Thus, native "active" protein is produced.

Representative examples of such therapeutic nucleic acid sequences are ones that code for Nerve Growth Factor (NGF), Brain Derived Growth Factor (BDNF), Ciliary Neurotrophic Factor (CNTF), Glial Cell Line-Derived Growth Factor (GDNF), Neurotrophin-3 (NT-3) or Neurotrophin 4/5 (NT-4/5).

The adenoviral system appears to be a very efficient system of production. This efficiency is due in part to the nature of adenoviral vectors. The adenoviral vectors transduce target cells at a high rate and copy number so that a large amount of recombinant gene is available for gene product expression. The adenoviral vectors cause the cells to stop all cellular gene expression and reallocate its biosynthetic machinery to express the vector genes. Thus, the entire cell is dedicated to produce the gene product. The vector is stable inside the cell so that gene product expression can take place for a long time.

Highly efficient promoters can be cloned into the adenoviral vectors so that the cloned gene is efficiently expressed at a high level.

The use of replication-defective adenoviral vectors and mammalian cells overcomes many of the problems associated with high level expression in bacteria. For example, in most bacteria high level production results in a product that is insoluble. Solubilization of these proteins from bacteria requires further purification which can lead to low or inactive yields. Production in bacteria precludes mammalian-cell specific post-translational modifications of the proteins. The use of adenoviral vectors avoids these problems. In the preferred embodiment, the strong promoter produces high concentrations of the bioactive molecule which is secreted from the cell into the medium. The bioactive molecule is easily purified since the medium composition can be controlled. The act of replacing genes in the adenoviral vectors that are required for viral replication with a nucleic acid cassette coding for a bioactive molecule renders the vector replication-defective, thus, it is safer than replication-competent viral vectors. The sum of the characteristics of vectors based upon adenoviruses add up to a vector system that is more efficient than current systems and permits post-translational modification.

In some instances mammalian cells' biosynthetic machinery is not sufficient alone to perform the necessary post-translational modification to yield native, bioactive protein. Some bioactive proteins

require further modification outside the producing cell. This type of post-translational modification is virtually impossible to reproduce in large-scale production processes due to the complexity. Transformation of cells in tissues of agricultural animals with adenoviral vectors permits the production of the recombinant protein in tissues that have the capacity to modify the protein with the organ's modification machinery.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

Example 1

Construction of the Adenoviral Vectors

Nucleic acid cassettes containing nucleotide sequences that code for bioactive molecules are inserted into the shuttle plasmid pADL.1 along with a promoter region. These plasmids are used along with pJM17 to transduce 293 cells that contain the adenovirus E1 gene region. Co-transduction resulted in homologous recombination where the promoter-nucleic acid cassette was exchanged for the E1 region in the pJM17 plasmid resulting in a vector that was replication defective and capable of directing the expression of the bioactive molecule by the host cells. In performing these manipulations vectors were generated that comprise the following elements operationally linked for functional expression; a promoter, a 5′ mRNA leader sequence; an initiation site; a nucleic acid cassette containing the sequence to be expressed; a 3′ untranslated region; and a

polyadenylation signal. The elements can be altered to modify the specificity and type of neuronal protective product that are generated.

Example 2

Production of Bioactive Molecule

To produce bioactive molecules cultured human cells were transduced with ADV-BDNF and ADV-CNTF at a ratio of 10 virus particles to cultured cell. The medium was replaced after 48 hours and 24 hours later the medium was removed and analyzed in the case of BDNF or the cells were analyzed in the case of CNTF. Figure 2 shows the results of Western blot analysis of the products from these transductions. The Western blot in Figure 2 shows that cells transduced with ADV-BDNF released BDNF into the medium that was molecularly equivalent to authentic BDNF (arrow). Cells transduced with ADV-βgal virus or not transduced did not produce BDNF as shown by the lack of an immuno-positive band. The Western blot in Figure 3 shows that cells transduced with ADV-CNTF made CNTF that was molecularly equivalent to authentic CNTF (arrow). Cells transduced with ADV-βgal virus or not transduced did not produce CNTF as shown by the lack of an immuno-positive band. The predicted production of neuronal protective factors was 1 mg per liter of culture medium. The rate was 10 micrograms/million cells/24 hours.

Example 3

Production of Bioactive Neuronal Growth Factor

To produce bioactive molecules cultured human cells were transduced with ADV-NGF at a ratio of 10 virus particles to cultured cell. The medium was

replaced after 48 hours and 24 hours later the medium was removed and analyzed in the cells. Figure 4 shows the production of a bioactive molecule by human cells after they were transduced with an adenoviral vector containing a nucleic acid cassettes coding for a bioactive molecule. The height of the bar represents the relative amount of bioactive molecule. The bars on the left show known amounts of the bioactive molecule NGF. The bars on the right show the amount of NGF in 1, 5, and 15 μl of conditioned medium removed from cultures of human cells that were transduced with ADV-NGF. Very high amounts of NGF were produced by the transduced cells.

Example 4

Biological Activity of Adenovirus Produced Proteins

Once experiments demonstrated that transduction of cells caused them to produce bioactive molecules it was important to demonstrate that the bioactive molecules had biological activity. Conditioned medium from 293 cell cultures that had been transfected with ADV-NGF was added to cultured PC12 cells to a final concentration of 10%. Control cultures contained medium from non-transfected 293 cells. PC12 cell cultures were then incubated 7 days with one change of medium after 4 days.

Figure 5 shows an example of the biological effect of an recombinant adenoviral vector containing the nucleic acid cassette for a bioactive molecule on rat pheochromocytoma cells (PC12 cells). In the presence of this bioactive molecule PC12 cells grow out projections that resemble and function as neuronal axons.

Extensive neurite outgrowth produced by the conditioned medium from ADV-NGF transfected cells indicated that the cells are responding to the biologically active vector product.

Example 5

Neuro-Protective Activity of Bioactive Molecules

After it was shown that bioactive molecules were released from cells transduced with adenoviral vectors containing nucleic acid cassettes for neuronal protective factors it was important to demonstrate the neuronal protective activity of the bioactive molecules on true neurons. A standard neuronal protective assay uses sensory neurons from embryonic chickens. In the absence of neuronal protective factors embryonic neurons die if removed from the embryo and placed in tissue culture.

Figure 6 shows the neuronal protective effect of the product of recombinant adenoviral vectors containing neuronal protective factors on embryonic chicken sensory cells. In this experiment human cells in culture were transduced with adenoviral vectors carrying the nucleic acid cassettes coding for BDNF, CNTF, and NGF. The culture medium was removed from the transduced cells and incubated with sensory neurons removed from embryonic chicken dorsal root ganglia at concentrations of 0.4, 2.0, 10, and 50%. Three days later the number of surviving neurons was counted and compared to the number surviving without neuronal protective factors (con). Very few or no neurons survived when they were grown in the presence of conditioned medium from non-transduced cells (con). In

the presence of conditioned medium from cells that were transduced with ADV-BDNF, ADV-NGF, and ADV-CNTF the neurons survived. The level of survival was related directly to the amount of the neuronal protective factors in the conditioned medium used to grow the cells. These results demonstrate that cells transduced with adenoviral vectors containing nucleic acid cassettes coding for bioactive molecules produced biologically active neuronal protective factors.

Example 6

Large-Scale Production of a Bioactive Molecule

Human HeLa cells (10^9 cells) are grown in a bioreactor containing 3 liters of medium. ADV-NGF is added at a ratio of 10 active particles to each cell (10^{10} active particles). The following days the medium is removed and the bioactive protein is isolated from the culture medium using standard large-scale protein isolation technology. The cultured cells are replenished with culture medium so that the cells can continue to produce NGF. The process is repeated until the capacity of the bioreactor is overburdened with HeLa cells. The yield is 15-20 mg of biologically active NGF a day.

Example 7

Large-Scale Production of a Bioactive Molecule in Tissues of Animals

ADV-NGF is injected into the muscle of a pig. Seven days later the pig is slaughtered and the bioactive molecule is isolated from the tissue.

Example 8

Large-Scale Production of a Bioactive Molecule in Bird Embryos

ADV-NGF is injected into fertilized chicken eggs. The eggs are incubated for 15 days and the NGF is isolated from the embryo and egg tissues.

Example 9

Large-Scale Production of a Bioactive Molecule in the Milk Glands of Animals

ADV-NGF is injected into the mammary glands of a lactating cow. NGF is isolated from the milk produced by the transduced mammary gland tissue.

Example 10

Large-Scale Production of a Bioactive Molecule in the Serum of Animals

ADV-NGF is infused into the blood supply of pigs. NGF is isolated from the serum from the animals.

Example 11

Adv.RSV-*nf* Construction

CNTF, BDNF, and GDNF cDNAs were synthesized by RT-PCR from total RNA from rat sciatic nerve, rat brain and the rat C_6 astrocytoma cell line, respectively, and cloned into pADL.1/RSV. Adv.RSV-*nf* vectors were generated by homologous recombination of the pADL.1/RSV constructs and pJM17 in HEK293 cells.

Example 12

NF Expression *In Vitro*

HeLa cells were transduced with Adv.RSV-*nf* at a multiplicity of infection (MOI) of 100. The medium was

replaced after 16 hr with serum-free DMEM, incubated for another 56 hr and then concentrated 10-fold with a 3000 molecular weight cut-off filter. For expression of CNTF, cells were harvested into Hank's balanced salt solution (HBSS), freeze-thawed and centrifuged to remove cellular debris. Control cells were transduced with an adenoviral vector carrying the gene for β-galactosidase (Adv.RSV-βgal). The conditioned medium or cell extracts were analyzed by Western blotting analysis using commercially obtained NF as standards and antibodies specific for NGF, BDNF, GDNF, and CNTF.

Example 13

Neuroprotection *In Vitro*

HeLa cells were transduced with the Adv.RSV-*nf* vectors at an MOI of 100 for 48 hr and the medium was replaced. After 24 hr the conditioned medium was collected (except for HeLa cells transduced with Adv.RSV-CNTF that were homogenized in HBSS as described above) and was analyzed for NF activity. Primary cultures of dissociated E10 chick embryo DRG or E6 NG were grown in DMEM with 10% fetal bovine serum that was supplemented with NF at 100 ng/ml for all NF except for 10 ng/ml of CNTF or 25% v/v of the conditioned media. In combination experiments the final concentration of the mix equaled 25% v/v. The numbers of surviving neurons in treated and in control cultures were determined by counting cresyl violet-stained neurons.

Example 14

Production of a Bioactive Molecule in HeLa Cells

Adv.RSV-*nf* were constructed that carried cDNAs coding for BDNF, CNTF, GDNF, and NGF under control of

the Rous sarcoma virus long terminal repeat (RSV). When HeLa cells were transduced with Adv.RSV-*nf* they produced proteins that were identical to NF standards in Western blot analyses (Fig.7). In the case of Adv.RSV-GDNF the presence of higher molecular weight cross-reacting species suggested that the protein was post-translationally modified, presumably by glycosylation. Cells transduced with Adv.RSV-NGF produced NGF at a rate of approximately 5 $\mu g/10^6$ cells/ day comparing favorably to estimates of 1 μg NGF/10^6 cells/day produced in cultured fibroblasts transduced with a herpes virus vector carrying a NGF cDNA. Estimates from Western blots of the expression rates of NF produced by cells transduced with the other Adv.RSV-*nf* suggested that the other NF were produced at similar rates.

The ability of the NF produced by HeLa cells transduced with Adv.RSV-*nf* to sustain the survival of embryonic sensory neurons was studied in chick nodose ganglia (NG) and dorsal root ganglia (DRG). The conditioned media had significant ($p<0.05$) survival effects compared to controls except for medium from cells transduced with Adv.RSV-NGF when applied to NG cells (Fig. 8). The conditioned media had greater biological effects than commercially available recombinant NF used at the optimal concentration recommended by the suppliers and suggests that the NF in the conditioned media may have greater specific activities than bacteria-derived NF, perhaps as a result of post-translational modification. Combining conditioned media from HeLa cells transduced with

different Adv.RSV-*nf* had additive survival effects (Fig. 8) as observed by others.

Ones skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The methods and procedures, molecules, cell lines and specific compounds described herein are presently representative of preferred embodiments, are exemplary and are not intended as limitations on the scope of the invention. Changes herein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the claims.

Claims

1. A method of producing a bioactive molecule, comprising the steps of:

introducing an adenoviral vector directly into cultured mammalian cells wherein the vector is comprised of a DNA sequence encoding a protein operatively linked to a promoter and wherein said vector produces the protein and said mammalian cells modified said protein to yield said bioactive molecule;

releasing said bioactive molecule into growth media or storing the bioactive molecule in the cells; and

isolating said bioactive molecule.

2. A method of producing a bioactive molecule comprising the steps of:

introducing an adenoviral vector directly into an animal wherein the vector is comprised of a DNA sequence encoding a protein operatively linked to a promoter and wherein said vector produces said protein, and cells in said animal modify the proteins to yield a bioactive molecule;

releasing said bioactive molecule into the fluid of an animal or storing the bioactive molecule in the cell or organ of the animal; and

isolating the bioactive molecule from said animal fluid, cell or organ.

3. The method of Claim 1 or 2 wherein the bioactive molecule is selected from the group consisting of Fibroblast Growth Factor, Endothelial Growth Factor, angiostatin, Epithelial Growth Factor, Vasopressin, Insulin, Hepatocyte Growth Factor, Insulin-Like Growth Factor, cytokines, Human Growth

Factor, Nerve Growth Factor, Brain Derived Growth Factor, Ciliary Neurotrophic Factor, Glial Cell Line-Derived Growth Factor, Neurotrophin-3 and Neurotrophin 4/5 (NT-4/5).

4. The methods of Claim 1 or 2 wherein the bioactive molecule is a therapeutic molecule selected from the group consisting of Nerve Growth Factor, Brain Derived Growth Factor, Ciliary Neurotrophic Factor, Glial Cell line-Derived Growth Factor, Neurotrophin-3 and Neurotrophin 4/5 (NT-4/5).

5. The method of claim 1 or 2 wherein the bioactive molecule acts upon the nervous system.

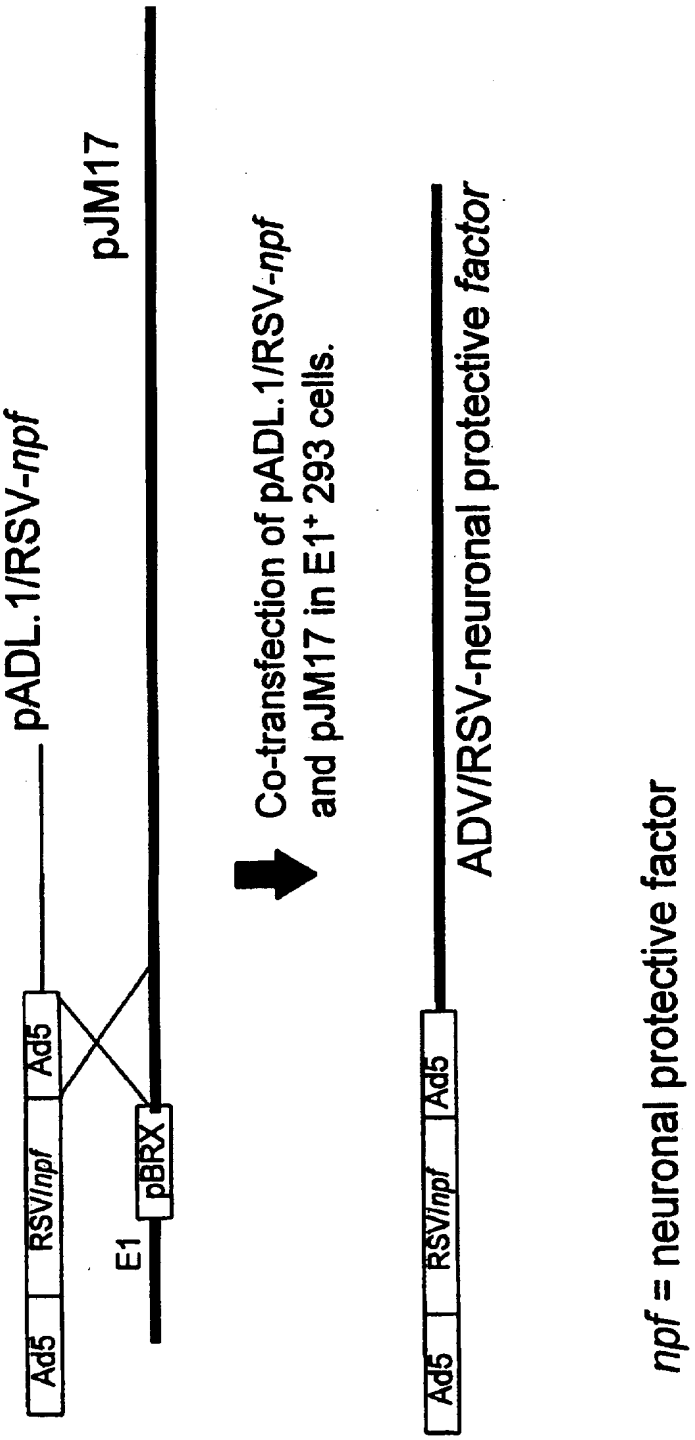
pADL.1/RSV-npf
pJM17
Ad5
RSVnpf
Ad5
E1
pBRX
Co-transfection of pADL.1/RSV-npf
and pJM17 in E1+ 293 cells.
ADV/RSV-neuronal protective factor
Ad5
RSVnpf
Ad5
npf = neuronal protective factor

Figure 1

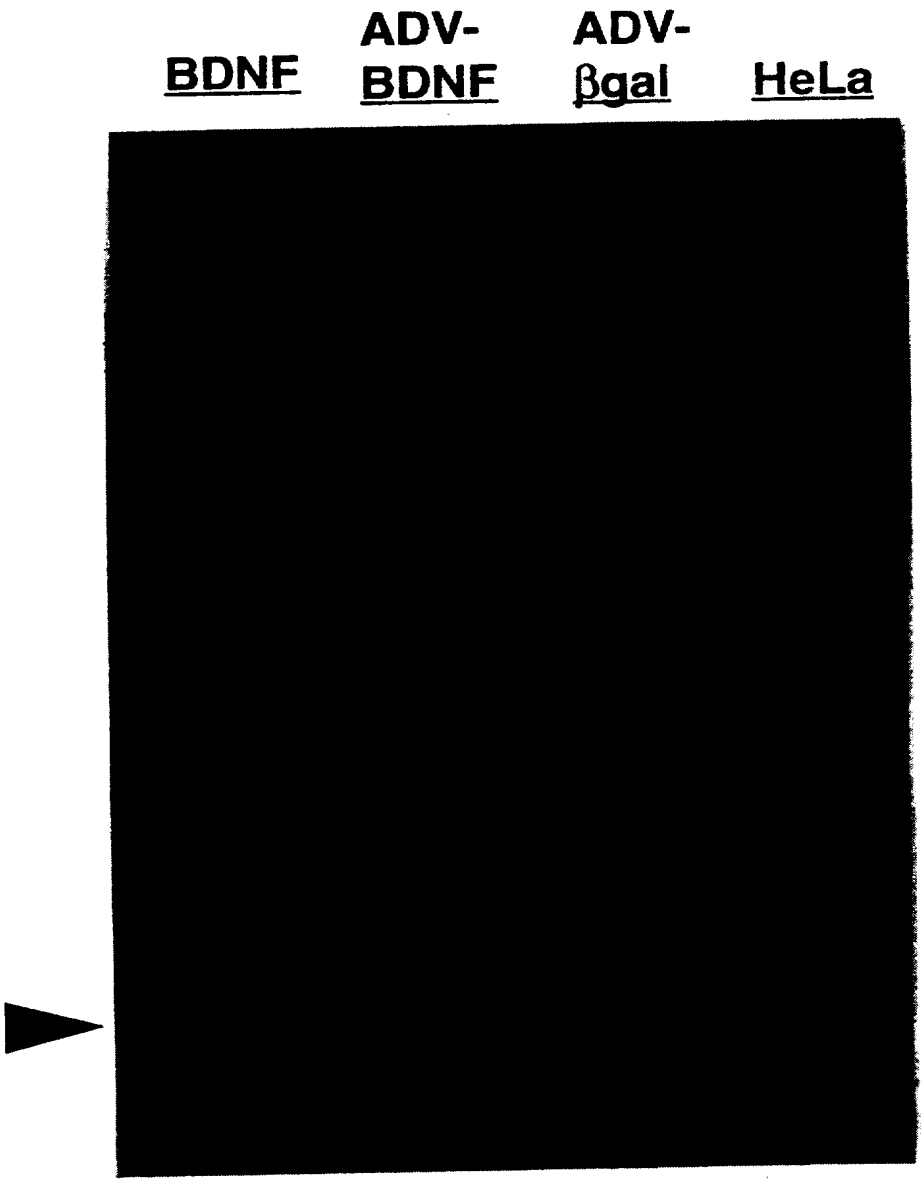

Figure 2

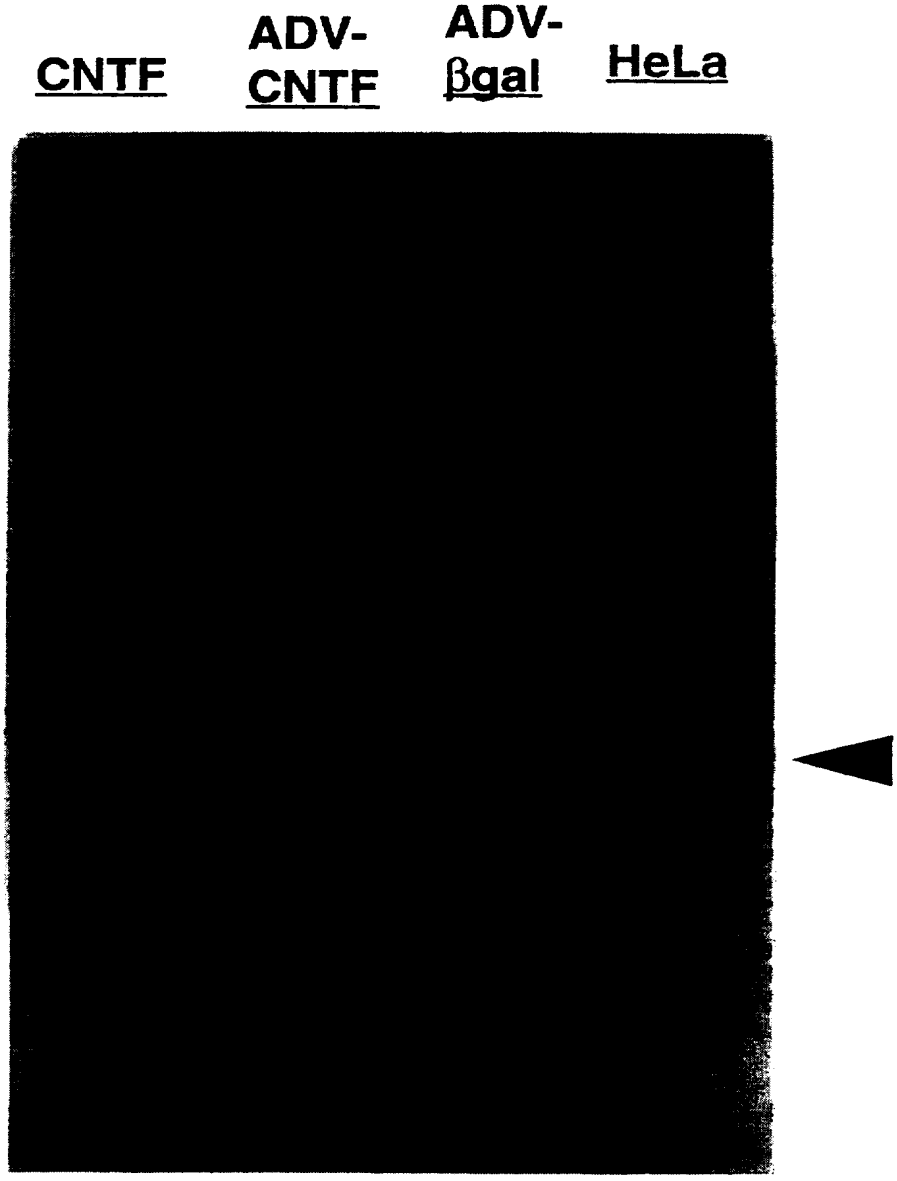

Figure 3

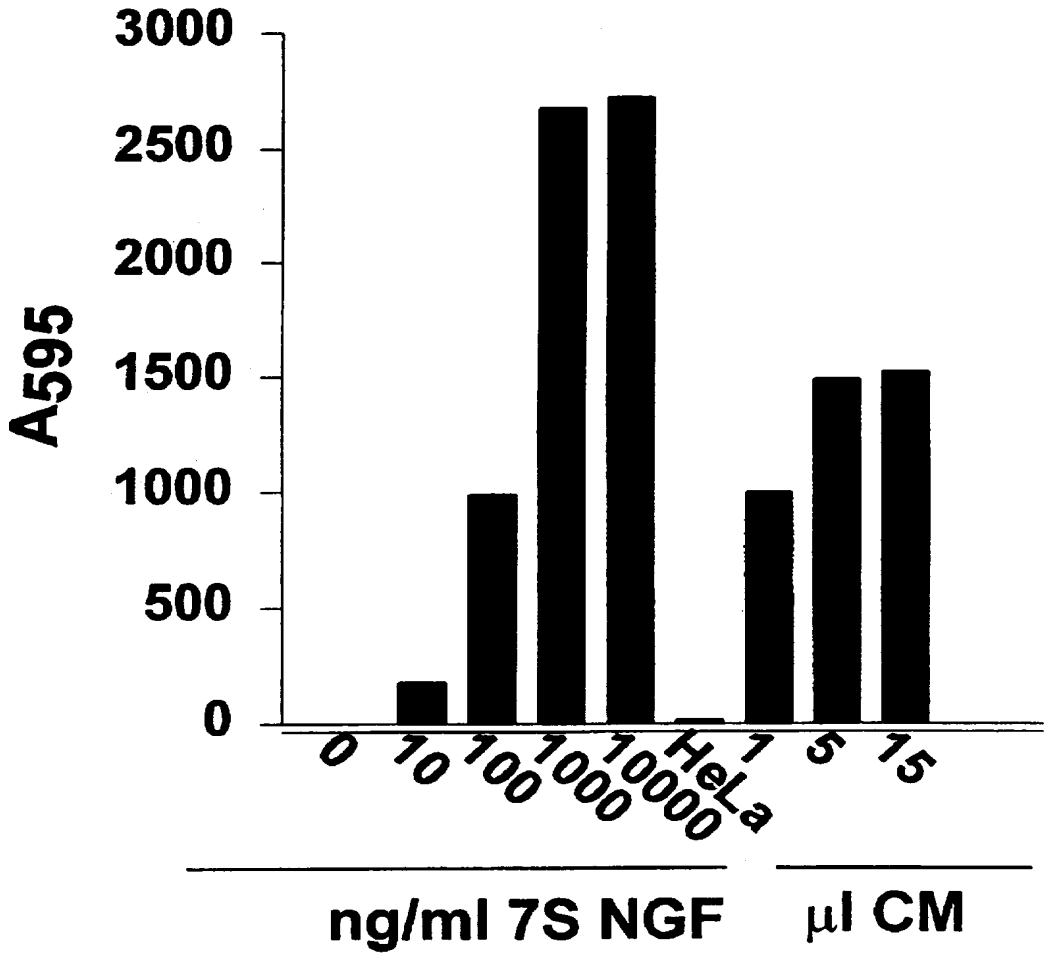
3000
2500
2000
1500
1000
500
0
A595
0
10
100
1000
10000
HeLa
1
5
15
ng/ml 7S NGF
µl CM

Figure 4

Figure 5A

Figure 5B

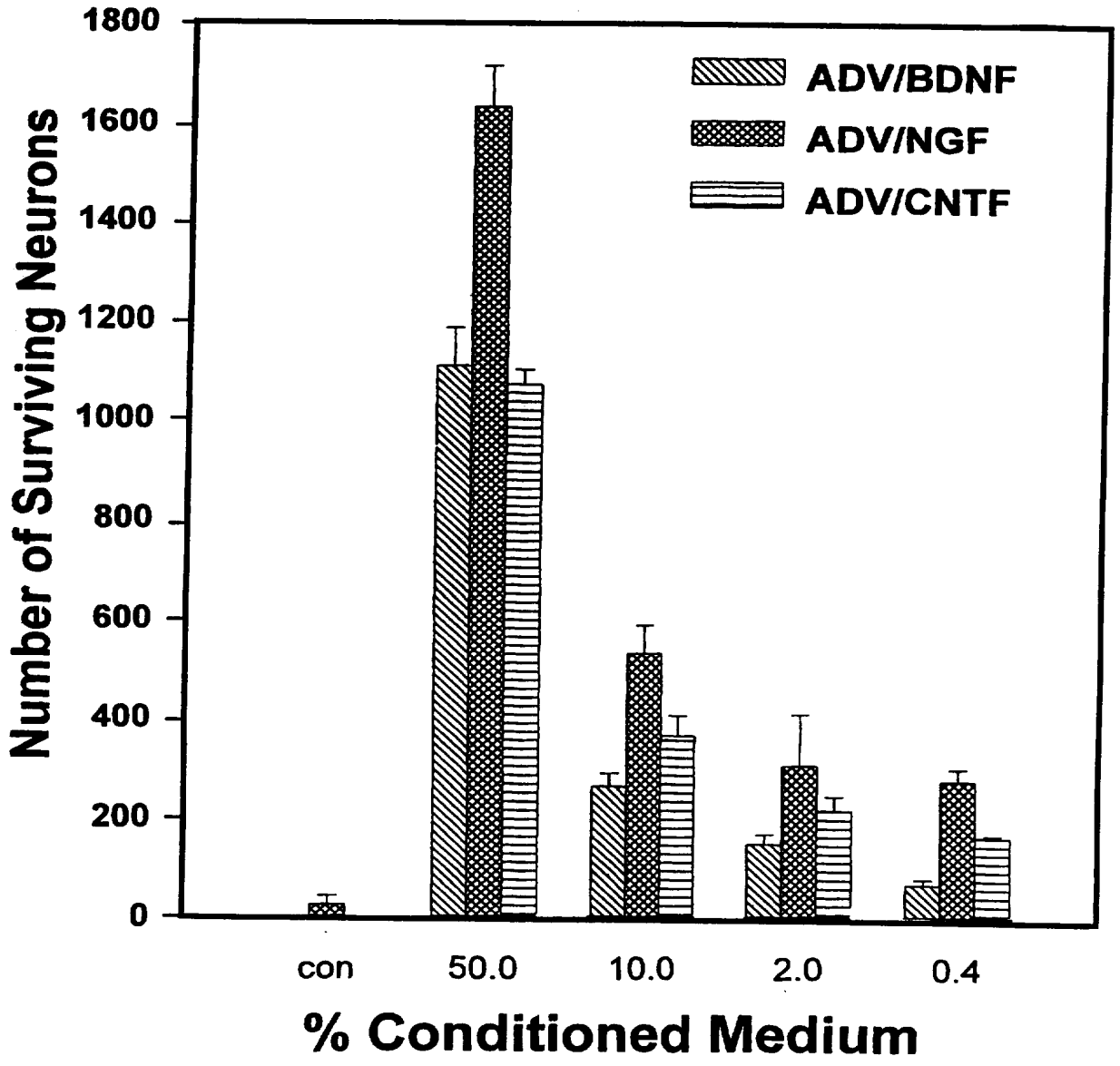
1800
1600
1400
1200
1000
800
600
400
200
0
Number of Surviving Neurons
ADV/BDNF
ADV/NGF
ADV/CNTF
con
50.0
10.0
2.0
0.4
% Conditioned Medium

Figure 6

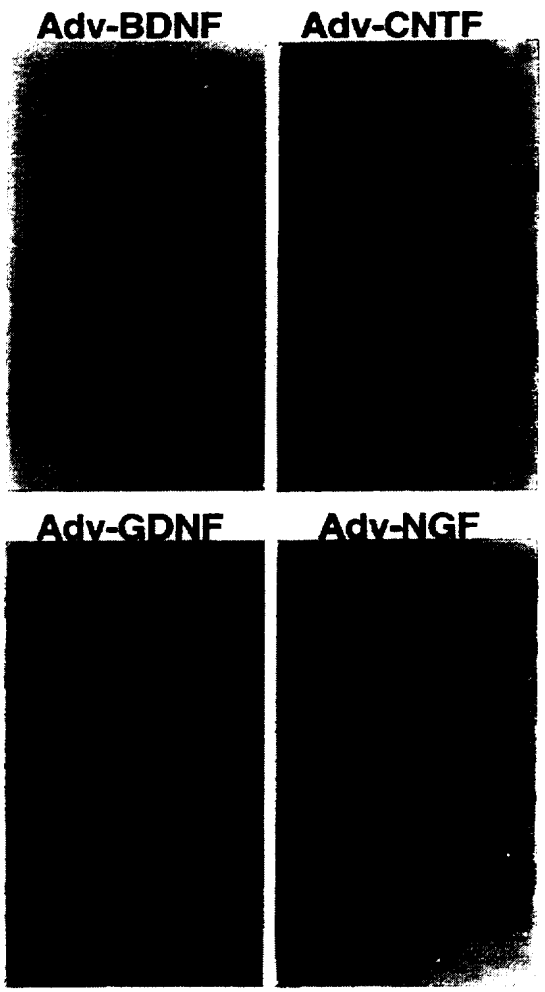

Figure 7

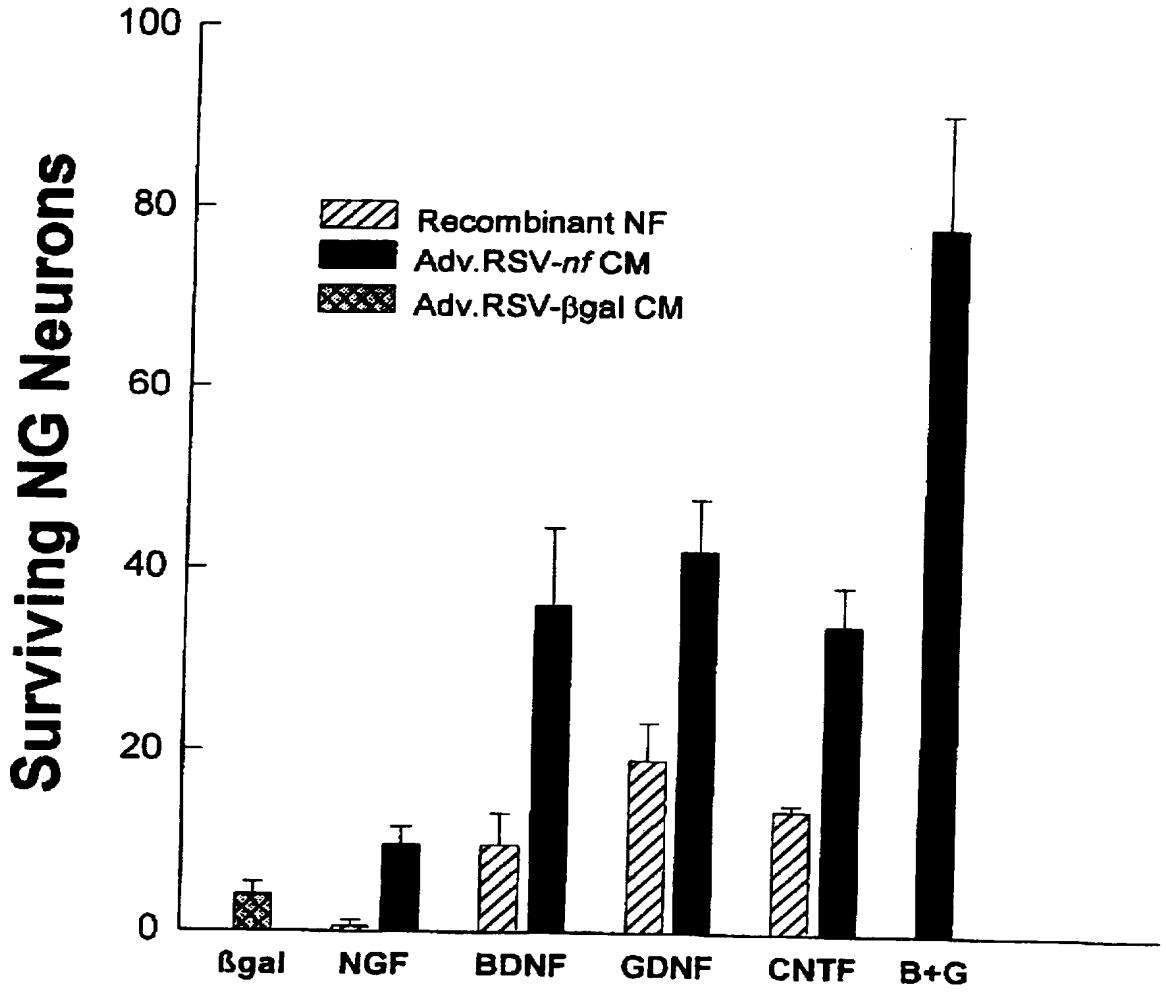
Surviving NG Neurons
100
80
60
40
20
0
Recombinant NF
Adv.RSV-nf CM
Adv.RSV-βgal CM
ßgal
NGF
BDNF
GDNF
CNTF
B+G

Figure 8A

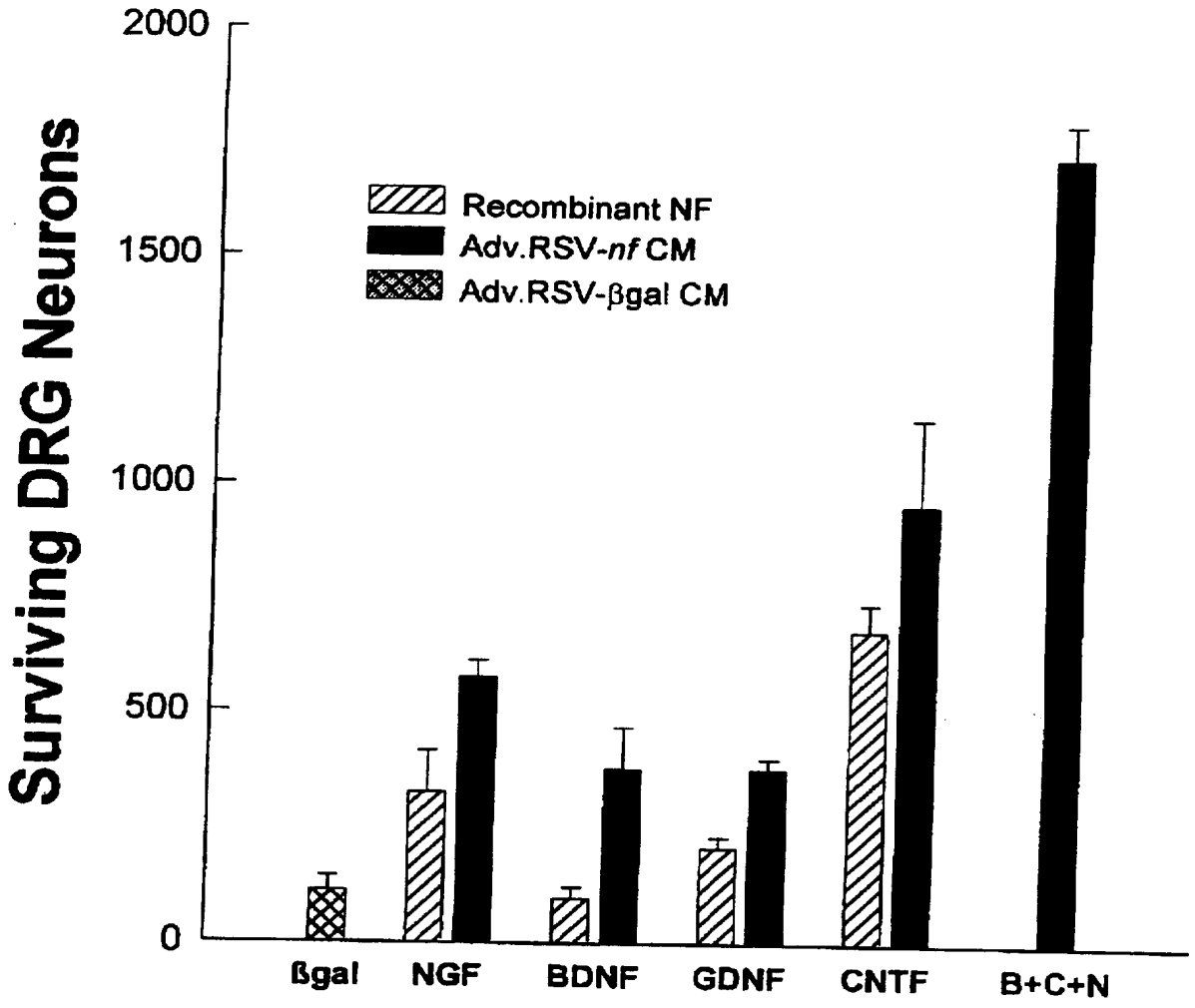
Surviving DRG Neurons
2000
1500
1000
500
0
Recombinant NF
Adv.RSV-nf CM
Adv.RSV-βgal CM
ßgal
NGF
BDNF
GDNF
CNTF
B+C+N

Figure 8B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17394

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 5/00, 15/00
US CL :514/44; 435/172.3, 320.1; 424/93.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/172.3, 320.1; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS MEDLINE BIOSIS EMBASE CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/08026 A1 (RHONE-POULENC RORER) 14 April 1994, see enitre document.	1-5
X	HORELLOU et al., "Direct Intracerebral Gene Transfer of an Adenoviral Vector Expressing Tyrosine Hydroxylase In a Rat Model of Parkinson's Disease", Neuroreport, Vol. 6, No. 1, 30 December 1994, pages 49-53, see entire document.	1-5
Y	NEVE et al., "Adenoviral Vectors Enter the Brain", Trends in Neuroscience, 1993, Volume 16, Number 7, pages 251-253, see entire document.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
06 JANUARY 1997

Date of mailing of the international search report
06 FEB 1997

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17394

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEDEROFF et al., "Expression of Nerve Growth Factor In Vivo From a Defective Herpes Simplex Virus 1 Vector Prevents Effects of Axotomy On Sympathetic Ganglia", Proceedings of the National Academy of Sciences, USA, March 1992, Volume 89, pages 1636-1640, see entire document.	1-5